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MOLECULAR IDENTIFICATION OF ONION YELLOW DWARF VIRUS IN *ALLIUM FISTULOSUM* AND *ALLIUM SCHOENOPRASUM* IN INDONESIA

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A B S T R A C T

Virus infection in four edible *Allium* spp.: Welsh onion (*Allium fistulosum*), onion (*Allium cepa*), fresh chive (*Allium schoenoprasum*), and garlic chive (*Allium tuberosum*) cultivated in Indonesia had never been studied. Samples were obtained during field surveys in June – October 2023 at different production centers in Java Island, Indonesia, and then molecularly tested using a universal primer pair to amplify ± 700 bp of the 3'-end region of *potyviruses*. Six Welsh onion and four fresh chive samples were tested positive for Onion yellow dwarf virus (*OYDV*). Phylogenetic tree clustered the sequenced four Indonesian Welsh onion isolates (GenBank acc. no. OR772075 – 78) in a group together with other Welsh onion isolates from South Korea and Japan. Meanwhile, the four fresh chive isolates (OR772079 – 82) that were obtained were positioned in a separate group together with predominantly onion isolates from Europe and America. No significant recombination signal was detected on the compared isolates using Recombination Detection Program v5.30. Percentage identity analysis further determined the divergence of Welsh onion isolates to isolates from other hosts.

Keywords: molecular detection, *Allium*, phylogenetic study, recombination analysis, Onion yellow dwarf virus.

INTRODUCTION

Besides shallot (*Allium cepa* var. *aggregatum*) and garlic (*Allium sativum*), many Indonesian foods are also extensively using onion, local name 'bawang Bombay' (*Allium cepa*) and Welsh onion, local name 'bawang daun' (*Allium fistulosum*) as main ingredients. On the other hand, garlic chives, local name 'bawang kucai' (*Allium tuberosum*) and fresh chives, local name 'bawang batak' or 'bawang lokio' (*Allium schoenoprasum*) are rarely used, mainly in some menus prepared by Chinese-Indonesian, thus grown limited in several areas of Indonesia.

Allium spp. have been widely reported to be subjects of infection by viruses from genera *Tospovirus*, *Potyvirus*, *Allexivirus*, and *Carlavirus* (Bag *et al.,* 2015; Santosa and Ertunc 2020; Cremer *et al.,* 2021). Mixed infection of some of these viruses is often called 'garlic viral complex', and accumulation of the viruses produced severe diseases and great losses up to 49% (Fajardo *et al.,* 2001; Conci *et al.,* 2003).

The full genome of members of *Potyvirus* is around 10 kbp in length, having one main Open Reading Frame (ORF) encoding one polyprotein that subsequently can be divided into coat protein (CP) and other nine small proteins (Verma *et al.,* 2015; Gupta *et al.,* 2017). Mansouri *et al.* (2021) detected at least one of three species of *potyviruses*: onion *yellow dwarf virus (OYDV), leek yellow stripe virus (LYSV)*, and *shallot yellow stripe virus (SYSV)* in onion, garlic, Welsh onion, fresh chives, and garlic chives during large-scale surveys in Czech Republic. Long before, *SYSV* isolates (GenBank acc. no. AB000472 and AB000473) were obtained from Welsh onion cultivated in Japan (Tsunesyoshi *et al.,* 1998). Previous studies also

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identified *OYDV*, *LYSV*, and *SYSV* in shallot and garlic in Indonesia (Harti *et al.,* 2020; Nurenik *et al.,* 2021; Hidayat *et al.,* 2023). However, no reliable reports on the viruses in other cultivated *Allium* spp. in the country, further emphasizing the lack of knowledge on the viruses infecting edible *Allium* spp. other than onion and garlic anywhere else.

In this study, onion, Welsh onion, fresh chives, and garlic chives samples were collected from central production areas in Java Island, Indonesia then subjected to RT-PCR test using a universal primer pair to detect *potyviruses*. Phylogenetic and diversity analyses was also performed to comprehend genetic relationship of the obtained new isolates with other isolates from various hosts and countries listed in NCBI GenBank. Therefore, the obtained results could contribute to our understanding on the *potyviruses* in different *Allium* spp. at global scale.

MATERIALS AND METHODS

Field surveys: Four *Allium* spp. were surveyed during field trips to different cultivation areas in June – October 2023. Welsh onion, which is planted widely, were sampled from three provinces in Java Island, Indonesia. Meanwhile, onion, fresh chives, and Welsh chives cultivations are limited to some spots only in the country. Fresh chives were sampled in West Java Province while garlic chives in Central Java Province and Yogyakarta Special Region. Onion samples were taken from Central Java Province. Samples were kept inside refrigerators (4 °C) in Plant Virology Laboratory, Universitas Gadjah Mada until tests.

Molecular identification: Small part of bulb or leaf of samples were cut then total RNA was extracted from them using 'Plant Total RNA Mini Kit' according to protocols provided by its manufacturer (Geneaid Biotech Ltd., Taiwan). Reverse transcription to synthesis cDNAs was done using ReverTra Ace kit' (Toyobo, Japan), each was in a reaction volume of 10 µl: 2 µl RNA, 1 µl dNTP, 0.5 µl (10 pmol μl^{-1}) Poty 1 primer (5′-GGATCCCGGGTTTTTTTTTTTTTT TTV-3′) (Gibbs and Mackenzie, 1997), 0.5 µl RNAse inhibitor, 0.5 µl ReverTraAce®', 2 µl 5× RT Buffer, and 3.5 µl nuclease-free water. The temperature cycle was programmed at 42 °C for 20 min, followed by 99 °C for 5 min.

The synthesized cDNAs were applied in subsequent PCRs, each was in a reaction volume of 40 µl: 4 µl of cDNA, 2 µl (10 pmol μl^{-1}) each of Poty 1 as the reverse primer and U341 (5′-CCGGAATTCATGR TITGGTGYATIGAIAAYGG-3′) as the forward primer for amplification of 600 - 800 bp of 3′-end of genome of *potyviruses* (Langeveld *et al.,* 1991), 20 µl of MyTaq HS Red Mix (Bioline, Germany), and 12 µl of PCR-grade double distillated water. The temperature cycle was programmed at 95 °C for 3 minutes (pre-denaturation), 35 cycles at 95 °C for 1 min (denaturation), 56 °C for 1 min (annealing) and 72 °C for 1 min (elongation), followed by 72 °C for 10 min (final elongation).

After electrophoresis for 50 min at 100 V, agarose gel stained with FloroSafe DNA Stain (1st BASE, Malaysia) was examined under UV light using a transilluminator (Optima Inc., Japan). Successfully amplified PCR products with the specific targeted band were submitted to a commercial company (1st BASE, Malaysia) to be bidirectionally sequenced using Sanger method. The recovered nucleotide sequences were examined using online software 'nucleotide BLAST' (https://blast.ncbi.nlm.nih.gov) to determine their genetically closest organism according to NCBI database. Sequences of the novel isolates were deposited in NCBI GenBank to secure specific accession numbers.

Recombination analysis: Nucleotide sequences of related isolates were downloaded from NCBI GenBank, aligned with the recovered sequences of new isolates, and then trimmed using ClustalW suit in MEGA11 v11.0.13 freeware (Tamura *et al.,* 2021). Possible recombinant event among aligned isolates was scanned by Recombination Detection Program (RDP v5.30) (Martin *et al.,* 2021). A recombination event was considered significant if it was supported by at least five of the algorithms implemented in RDP v5.30 (Bonferroni-corrected *P* value of < 0.05).

Phylogenetic and percentage identity analyses: Tamura-Nei-parameter model (Tamura and Nei, 1993) obtained the lowest Bayesian Information Criterion score thus confirmed as the most suitable one to construct the phylogenetic tree using Maximum Likelihood statistical method with 1000 bootstrap replicates implemented in MEGA11 v11.0.13 freeware (Tamura *et al.,* 2021). The percentage identities among sequences of the observed isolates at nucleotide (nt) and amino acid (aa) levels were calculated using Sequence Demarcation Tool (SDT v1.2) (Muhire *et al.,* 2014).

RESULTS

Field surveys: Field trips during June – October 2023 collected five fresh chives samples from Bandung Regency, West Java Province. Two garlic chives were taken from Semarang Regency, Central Java Province and three were from Sleman Regency, Yogyakarta Special Region. Four onion samples were obtained from Magelang Regency, Central Java Province. Two Welsh onion samples were collected from Karanganyar Regency, Central Java Province; two from Semarang Regency, Central Java Province; four from Sleman Regency, Yogyakarta Special Region; and two from Nganjuk Regency, East Java Province. The total number of obtained samples were 24 (Figure 1).

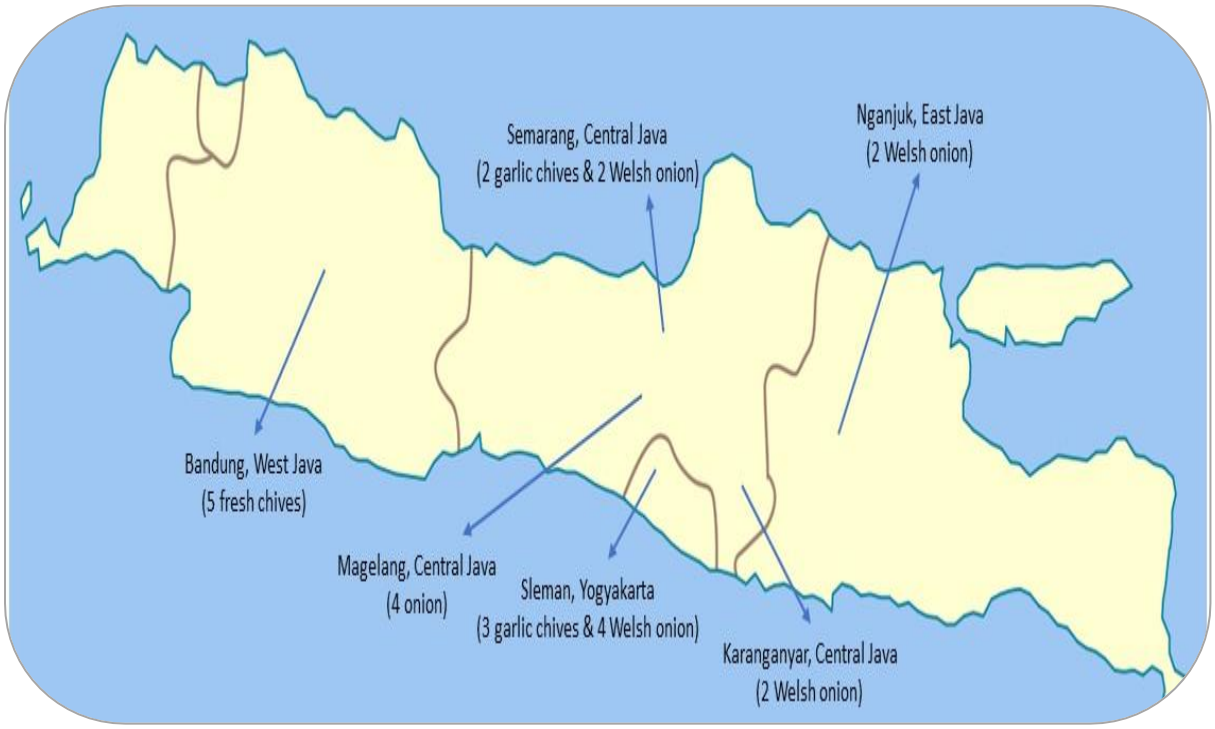

Figure 1. Sampling locations within Java Island, Indonesia, and numbers of the collected four *Allium* spp samples.

Molecular identification: Six Welsh onion and four fresh chives samples were tested positive for *potyvirus* infection by each formed a single band of ± 700 bps on agarose gel after RT-PCR. Other samples did not form any band on agarose gel thus tested negative for *potyvirus* infection (Table 1). The 3′-end region of four Welsh onion and four fresh chives isolates representing different origins were sequenced. Results of BLAST analysis showed that all eight of the obtained sequences had the highest similarity to *OYDV*. Sequences of eight new *OYDV* isolates from Indonesia were deposited in GenBank with acc. no. OR772075 - OR772082 (Table 1).

Recombination analysis: RDP5 analysis did not detect any significant recombination signals on the tested 3′-end CP region sequences of new Indonesian or isolates from other countries listed in NCBI GenBank.

Phylogenetic analysis: The phylogenetic tree analysis effectively categorized all the tested isolates into three significant clusters. Notably, the four fresh chive isolates from Indonesia were grouped in cluster 1, indicating their close relationship with four *Allium cepa* isolates from Argentina, Italy, and Germany, one shallot, and one Welsh onion isolate from Serbia. The study discovered that the four Welsh onion isolates from Indonesia belonged to cluster 3 and were closely related to South Korea and Japan isolates. The isolate from Semarang (OR772075) showed a coat protein sequence variation that differed from the other three isolates from Nganjuk, Sleman, and Karanganyar, as seen from the separated clade.

Percentage identity analysis: The 3′-end CP region of the compared *OYDV* isolates have 79.7 – 99.8% and 83.1 – 100% identities among them at nt and aa levels, respectively. The SDT analysis also estimated that the four new and two Welsh onion isolates in group 3 shared 88.3 – 98.9 % nt and 88.3 – 99.4% aa identities among them, and 79.7 – 88.3% nt and 83.1 – 94.2% aa identities to isolates from different hosts (Figure 3).

Table 1. Results of molecular identification of *potyviruses* in four *Allium* spp. samples collected in different locations within Java Island, Indonesia

Sample no.	Host species	Origin	RT-PCR result	Sequencing result	Isolate name	GenBank Acc. No.
1.	onion	Magelang, Central Java	-	-	-	-
2.		Magelang, Central Java	-	-	-	-
3.		Magelang, Central Java	-	-	-	-
4.		Magelang, Central Java	-	-	-	-
5.	fresh chives	Bandung, West Java	+	*OYDV*	W. Java:Bandung.1Os	OR772079
6.		Bandung, West Java	+	*OYDV*	W. Java:Bandung.2Os	OR772080
7.		Bandung, West Java	+	*OYDV*	W. Java:Bandung.3Os	OR772081
8.		Bandung, West Java	+	*OYDV*	W. Java:Bandung.4Os	OR772082
9.		Bandung, West Java	-	-	-	-
10.	garlic chives	Semarang, Central Java	-	-	-	-
11.		Semarang, Central Java	-	-	-	-
12.		Sleman, Yogyakarta	-	-	-	-
13.		Sleman, Yogyakarta	-	-	-	-
14.		Sleman, Yogyakarta	-	-	-	-
15.	Welsh onion	Semarang, Central Java	+	*OYDV*	C. Java:Semarang.1Of	OR772075
16.		Semarang, Central Java	+	not sequenced	-	-
17.		Nganjuk, East Java	+	*OYDV*	E. Java:Nganjuk.1Of	OR772076
18.		Nganjuk, East Java	-	-	-	-
19.		Karanganyar, Central Java	+	*OYDV*	C. Java:Karanganyar.1Of	OR772077
20.		Karanganyar, Central Java	-	-	-	-
21.		Sleman, Yogyakarta	-	-	-	-
22.		Sleman, Yogyakarta	+	*OYDV*	Yogyakarta:Sleman.1Of	OR772078
23.		Sleman, Yogyakarta	+	not sequenced	-	-
24.		Sleman, Yogyakarta	-	-	-	-

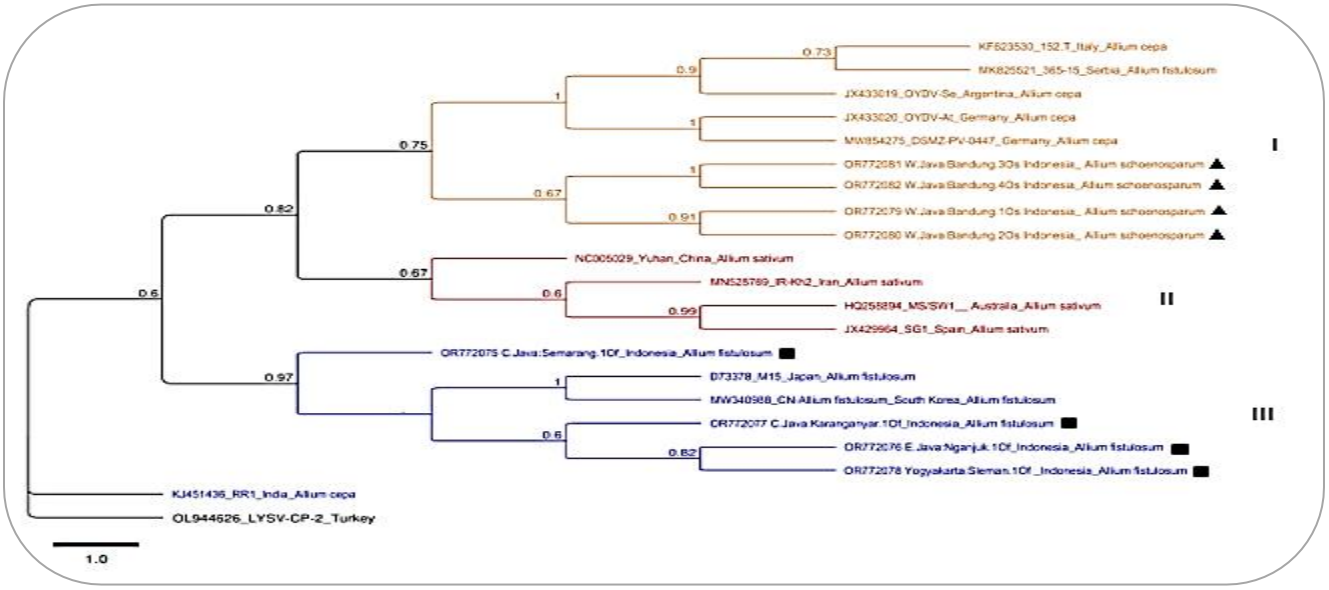

Figure 2. A phylogenetic tree based on complete 3′-end CP region (683 bp) of *OYDV* genome was constructed by MEGA11 software using Tamura-Nei-parameter model in Maximum Likelihood statistical method with 1000 bootstraps suited in. Only bootstrap values greater than 50% were shown. Black squares and triangles pointed the new welsh onion and fresh chive isolates characterized in this study, respectively.

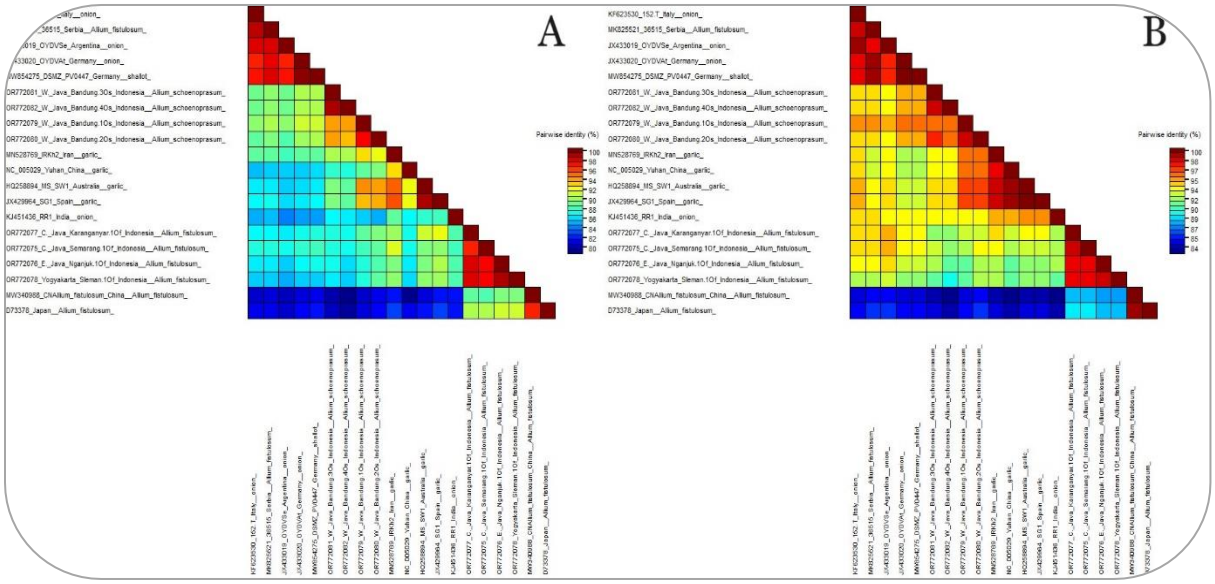

Figure 3. Percentage identities among compared *OYDV* isolates at A. nucleotide and B. amino acid levels were estimated using Sequence Demarcation Tool (SDT) v1.2.

DISCUSSION

Shallot and garlic cultivated in Indonesia have been shown to be widely infected by three *potyviruses*: *OYDV*, *LYSV*, and *SYSV*, with infection rates of up to more than 90% in some fields due to vegetative propagation of the two *Allium* spp. (Harti *et al.,* 2020; Nurenik *et al.,* 2021; Hidayat *et al.,* 2023). However, there is no clear indication whether other edible Allium in the country is also infected by *potyviruses* or not. Information on the *potyviruses* in *Allium* spp. with small economic importance is similarly lacking on the Global stage.

A molecular test using the universal primer pair detected *potyvirus* in fresh chives and Welsh onion samples. *OYDV* was probably widespread among fresh chives as four of five samples were found to be infected. Onion and garlic chives samples were all tested negative, although both species have been reported to be susceptible to *potyviruses* in other studies (Mansouri *et al.,* 2021; Santosa *et al.,* 2023). Therefore, it is possible that *potyviruses* is not common in the surveyed areas, but still can be detected when additional onion and garlic chives samples from different regions of Indonesia are acquired in future studies.

These results also provided the first insights into genome of *OYDV* isolates infecting fresh chives and Welsh onion grown in Indonesia. *OYDV* had been identified in both *Allium* spp. in Czech Republic using serological and molecular methods but the study did not produce any sequence data (Mansouri *et al.,* 2021). Therefore, the acc. no. OR772075 - OR772082 of this current study were the first sequences of *OYDV* from fresh chives deposited in GenBank. Previous partial sequences of *OYDV* in Welsh onion were from Japan (acc. no. D73378), Serbia (MK825521), and South Korea (MW340988) but they seem to be not analyzed further and published.

Phylogroupings of the tested isolates in this study were observed to be largely based on host species: onion (group 1), garlic (group 2), and Welsh onion (group 3). The obtained fresh chives isolates belong to group 1. Indonesian Welsh onion isolates showed a close relationship with other Welsh onion isolates from South Korea and Japan in group 3. Results of percentage identity analysis gave further evidence that Welsh onion isolates are highly variable to other compared isolates. These, combined with the fact that there is no recombination in the genome of the tested isolates, suggested that some *OYDV* isolates adaptation to Welsh onion led to divergence into a separate lineage from those isolated from other host species. However, the fresh chive isolate (MK825521) is grouped with fresh chive and onion isolates in group 1, while the onion isolate (KJ451436) is in a separate clade.

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Aprilia S. Subiastuti	:	Data analysis, writing and revising the manuscript, and corresponding author